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EFFECT OF AMMONIUM CHLORIDE ON RECEPTOR-MEDIATED UPTAKE OF DIPHTHERIA TOXIN BY VERO CELLS

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SUMMARY

The mechanism of NH$_4$Cl-mediated protection of Vero cells from diphtheria toxin was studied. In the presence of protective concentrations of NH$_4$Cl, Vero cells bound, internalized, and degraded radiolabeled diphtheria toxin at the same rate and to the same extent as did the control cells. However, in experiments where specific antibody was added to NH$_4$Cl-treated cells, a fraction of potentially lethal toxin molecules was maintained in a position accessible to antibody neutralization. This suggests the existence of two processing mechanisms for diphtheria toxin: a non-productive bulk degradation pathway and a productive NH$_4$Cl-sensitive pathway by which active fragment is eventually delivered to the cytoplasm.

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Diphtheria toxin, the 62 000 MW protein exotoxin secreted by certain strains of *Corynebacterium diphtheriae*, is toxic to a large number of cultured mammalian cell lines. Cytotoxicity results from the toxin-catalyzed inactivation of elongation factor 2 and the subsequent inhibition of intracellular protein synthesis [1]. To exert this cytotoxic effect, diphtheria toxin or its enzymatically active fragment must enter the cytoplasm. Recent evidence suggests that cytoplasmic entry is effected by adsorptive endocytosis [2].

Radiolabeled diphtheria toxin has been shown to bind to specific high-affinity receptors on the surfaces of cultured monkey kidney cells [3]. Receptor-bound toxin is rapidly internalized by cells (half-time of internalization, approx. 25 min), degraded, and excreted into the culture medium in the form of trichloroacetic acid (TCA)-soluble fragments [2]. The rapidity and extent of the degradation process imply the participa-

tion of lysosomes. Further support for lysosomal involvement is provided by the recent finding that both toxin degradation and toxin-induced inhibition of protein synthesis are blocked by the potent lysosomotropic agent, chloroquine [4]. This process of rapid receptor-mediated uptake followed by lysosomal degradation appears to correspond to the adsorptive endocytic mechanisms described for a number of other macromolecular uptake systems, including those responsible for cellular uptake of low-density lipoprotein [5], epidermal growth factor [6], and α$_2$-macroglobulin [7].

A number of drugs and chemicals have been identified which protect cells from toxin-mediated cytotoxicity [8]. One of the most effective protective agents is ammonium chloride [8, 9]. It has been suggested that NH$_4$Cl acts by maintaining diphtheria

toxin on the cell surface in a position amenable to neutralization by specific antibody [9, 10]. In this paper, we present results demonstrating that protective levels of NH₄Cl do not prevent bulk uptake of diphtheria toxin by receptor-mediated endocytosis. It therefore appears possible that two cellular processing mechanisms for diphtheria toxin exist: a non-productive bulk uptake and degradation pathway and a productive NH₄Cl-sensitive pathway that leads to the release of active fragment A.

MATERIALS AND METHODS

Cells and cell cultures

Seed stock for Vero cells was obtained from the American Type Culture Collection (ATCC). Rockville. Md. The cells were maintained in 75-cm² T-flasks (Costar no. 3075) with the medium and serum supplement recommended by ATCC.

Media and sera

All media. vitamins. amino acids. and antibiotics were obtained from Gibco. Grand Island. NY. Fetal calf serum (FCS) was purchased from Reheis Chemical Co.. Phoenix. Ariz. The serum was heat-inactivated for 30 min at 56°C before use in cell culture.

Toxin and antitoxin

Diphtheria toxin was obtained from Connaught Laboratories. Toronto. and purified by chromatography over DE-52 (Whatman). Toxin concentration was determined using an extinction coefficient $(E_{1cm}^{1\%})$ at 280 nm of 11.9.

Radiolabeled toxin was prepared as previously described [3] by the method of Roth [11]. Labeling routinely proceeded to the level of 1–2×10⁷ cpm/μg (0.1–0.2 mol iodine/mol toxin) and the labeling procedure had no detectable effect on the biological activity of the toxin, as measured by cytotoxicity assay.

Diphtheria toxin-specific antiserum, obtained by hyperimmunization of a goat, was prepared using purified diphtheria toxoid obtained from Dr R. Holmes. Uniformed Services University of Health Sciences.

Protein synthesis assay

Cells were seeded in 24-well tissue culture plates (Costar no. 3524) and grown to a concentration of 1–2×10⁵ cells/well. On the day of experimentation. the medium was replaced with 0.4 ml/well of Hanks' 199 (H199) supplemented with 5% FCS and 25 mM Hepes, pH 7.4 (complete H199). and cells were equilibrated

to 37°C. Ammonium chloride and diphtheria toxin were added in 50-μl aliquots to the desired concentrations in triplicate samples. Two hours later. 1 μCi/ml of [³H]leucine (New England Nuclear) was added and incubation continued for 1–2 h. Cells were harvested by rinsing once with Hanks' balanced salt solution (HBSS) containing 1 mg/ml L-leucine and once with HBSS alone. The monolayers were then solubilized for 5–10 min in a freshly prepared 1:1 mixture of DNase 1 (Sigma). 0.5 mg/ml in 0.05 M Hepes. 2 mM CaCl₂. and 2 mM MgCl₂. and 0.15% sodium dodecylsulfate (SDS). Solubilized samples were absorbed on numbered 11-mm paper discs (Schleicher & Schuell. no. 740E). and precipitated by the addition of 1 ml/well of 10% trichloroacetic acid (TCA). The discs then were collected in a bottle and washed twice with 5% TCA. twice with 1:1 ethanol : ether. and once with ether. After drying. the discs were counted in a toluene-based liquid scintillation solution (Liquifluor. New England Nuclear).

Cytotoxicity assay

Details of the cytotoxicity assay have been described previously [8]. Cells were seeded in 24-well tissue culture plates and grown to a concentration of 1–2×10⁵ cells/well. Toxin was added to the desired concentration in 100-μl aliquots and incubation continued for 3 h at 37°C. The cells then were rinsed three times with unsupplemented Earle's 199 medium. fresh complete medium added. and incubation continued for a further 48 h. Monolayers then were rinsed twice with HBSS to remove detached cells and the remaining cells were solubilized in 0.5 ml/well 0.1 M NaOH. Solubilized samples were analyzed for protein content [12].

Binding assay

Details of the binding assay have been previously described [3]. Cells were seeded in 24-well tissue culture plates and grown to a concentration of approx. 1–2×10⁵ cells/well. On the day of experimentation. the medium was replaced with 1.0 ml of complete H199. ¹²⁵I-Diphtheria toxin (0.03 μg/ml) or ¹²⁵I-toxin plus a 100-fold excess of unlabeled toxin was added in 10-μl aliquots to the wells and incubations were carried out at the temperatures and for the times indicated. The monolayers were then rinsed three times with HBSS. solubilized in 1.0 ml of 0.1 M NaOH and counted in a Model 1185 automatic gamma counting system (Searle Analytic. Inc.).

The difference between binding of labeled toxin in the presence and absence of excess unlabeled toxin represents specific binding; the data presented in this paper are shown in the form of specific counts. The level of non-specific binding in all cases represented less than 10% of the total.

Internalization assay

The internalization assay has been previously described [2]. Cell monolayers were incubated with ¹²⁵I-toxin with or without a 100-fold excess of unlabeled toxin as described above. The monolayers then were rinsed three times with cold HBSS. fresh

complete medium was added, and the cells were warmed to 37°C. At the indicated times, cells were rinsed once with cold HBSS and incubated 60 min at 4°C with 0.5 ml of pronase (Calbiochem no. 537088) (0.25 mg/ml) plus inositol hexaphosphate (PIHP) (10 mg/ml) in HBSS. At the end of the incubation period, 0.5 ml of heat-inactivated FCS was added to each well; the detached cells were immediately transferred to 1.5-ml polypropylene centrifuge tubes and spun for 1 min in a microfuge (Eppendorf Model 5412). Both pellets and supernatants were counted as above.

Degradation assay

The degradation assay has been previously described [2]. Cell monolayers were incubated with ^{125}I-toxin with or without a 100-fold excess of unlabeled toxin as described above. The monolayers then were rinsed three times with cold HBSS. 1 ml/well of fresh complete medium was added, and the cells were warmed to 37°C. At the times indicated, the culture medium was removed and added to an equal volume of cold 10% TCA in 12×75 mm glass tubes. The samples were mixed and centrifuged at 4°C for 10 min at 1200 g; supernatants and pellets were counted as above.

Autoradiography

Cells were incubated with ^{125}I-labeled toxin (0.1 μg/ml) or ^{125}I-labeled toxin plus a 100-fold excess of unlabeled toxin at 4°C for 4 h or at 37°C for 2 h. In the NH₄Cl-treated samples, cells were incubated with 10 mM NH₄Cl for 1 h at 37°C prior to toxin addition. The cells then were rinsed three times with HBSS, fixed with 2% glutaraldehyde, and processed for autoradiography as described by Gorden et al. [13]. After 21 days exposure, grids were developed and examined in a JEOL-JEM 100B electron microscope at ×10000 magnification.

RESULTS

Effect of NH₄Cl on toxin-induced inhibition of protein synthesis

The effect of NH₄Cl on diphtheria toxin-induced inhibition of protein synthesis in Vero cells is shown in fig. 1. In the absence of NH₄Cl, a 2-h incubation with 2 ng/ml toxin blocks protein synthesis 80–90%. In the presence of NH₄Cl (10 mM), however, the cells were completely protected from the cytotoxic effect. Comparable results were obtained in 3-h cytotoxicity assays (data not shown).

Fig. 1. Effect of NH₄Cl on diphtheria toxin-induced inhibition of protein synthesis. ○, Control; △, NH₄Cl. 10 mM.

Effects of NH₄Cl on uptake of radiolabeled diphtheria toxin

Previous work has shown that at 37°C the uptake of ^{125}I-labeled diphtheria toxin by Vero cells follows a biphasic pattern, increasing to a peak at 1½–2 h and subsequently decreasing to a steady state. This biphasic pattern is believed to result from a combination of (1) internalization and degradation of toxin with accompanying consumption of receptor; and (2) block of receptor reappearance on the cell surface due to toxin-induced inhibition of protein synthesis (unpublished data). In the presence of a protective concentration of NH₄Cl (fig. 2), the biphasic response is not observed and cell-associated radioactivity increases to a plateau level by about 3 h. NH₄Cl had no effect on ^{125}I-diphtheria toxin binding at 4°C (data not shown).

The experiment shown in fig. 3 demonstrates that the plateau attained at 37°C in the presence of NH₄Cl represents continuous toxin uptake. If excess unlabeled diphtheria toxin is added to cells that have reached the plateau level in the presence of a saturating concentration of ^{125}I-labeled toxin, cell-associated radioactivity decreases rapidly. This suggests that the un-
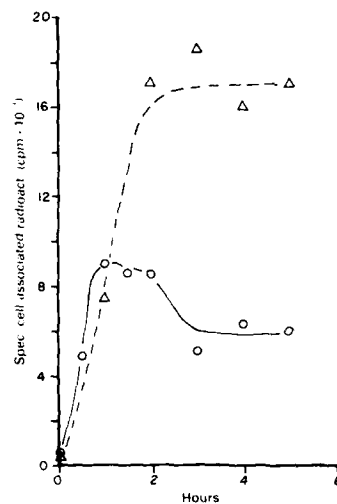
Fig. 2. Effect of NH₄Cl on the uptake of diphtheria toxin. ○, Control; △, NH₄Cl, 10 mM.

Fig. 4. Effect of NH₄Cl on the internalization of diphtheria toxin. ○, Control supernatant (cell-surface radioactivity); ●, control pellet (internalized radioactivity); □, NH₄Cl supernatant (cell-surface radioactivity); ▪, NH₄Cl pellet (internalized radioactivity).

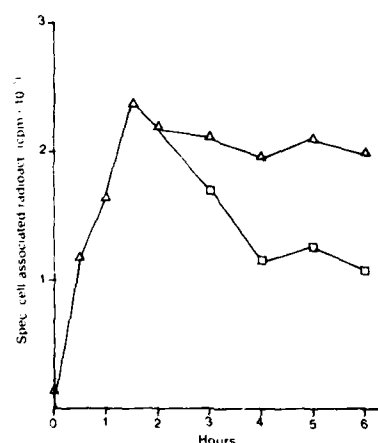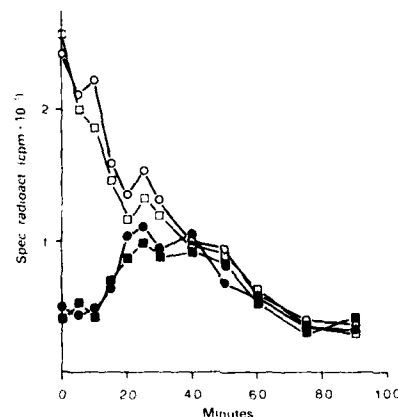labeled toxin molecules are able to compete effectively for newly available surface receptors and thus implies a steady-state rate of active uptake.

Fig. 3. Continuation of diphtheria toxin uptake in the presence of NH₄Cl. △, NH₄Cl, 10 mM; □, NH₄Cl, 10 mM + unlabeled toxin (3 μg/ml).

Effect of NH₄Cl on internalization and degradation of radiolabeled diphtheria toxin

We next investigated the effect of NH₄Cl on toxin internalization, using our previously described internalization assay [2]. Cells were preincubated at 4°C with ¹²⁵I-diphtheria toxin in the presence or absence of NH₄Cl, washed to remove unbound toxin, and warmed to 37°C, again in the presence or absence of NH₄Cl. Results (fig. 4) demonstrate that NH₄Cl had no effect on either the rate or extent of internalization. In both NH₄Cl-treated and control cells, the half-time of internalization was approx. 25 min and about 90% of cell-associated radioactivity was resistant to PIHP after 90 min. Parallel experiments showed that NH₄Cl had no effect on toxin degradation (fig. 5) as determined by measurement of TCA-soluble radioactivity excreted into the culture medium. Rate and extent of degradation were not altered by NH₄Cl; approx. 60–70% of the cell-associ-

Table 1. *Autoradiography of Vero cells treated with ^{125}I-diphtheria toxin and NH_4Cl*

Condition	No. cells	No. silver grains	Membrane-associated silver grains (%)	Cytoplasm-associated silver grains (%)
4°C				
Toxin only	149	275	60	40
37°C				
Toxin only	61	282	10	90
+NH₄Cl, 10 mM	64	441	3.5	96.5
+conA, 100 μg/ml	304	67	61	39

ated radioactivity was in TCA-soluble form by 3 h.

Autoradiography of NH₄Cl-treated cells

Since the previously described biochemical techniques strongly indicated that NH₄Cl did not prevent bulk toxin internalization, we next attempted to confirm these results by morphological methods. Cells were incubated with ^{125}I-labeled diphtheria toxin for 4 h at 4°C or 2 h at 37°C in the presence or absence of NH₄Cl. The cells then were washed with HBSS, fixed with 2% glutaraldehyde, and processed for electron microscopic autoradiography as described in Materials and Methods. Results are listed in

80

60

40

20

0 1 2 3 4 5
Hours

Fig. 5. Effect of NH₄Cl on the degradation of diphtheria toxin. ○, Control; △, NH₄Cl, 10 mM.

table 1. At 4°C, the toxin molecules were bound at the cell surface and the majority of the silver grains (60%) were associated with the cell membranes. After a 2-h incubation at 37°C, however, most of the surface-bound toxin had been internalized; only 10% of the silver grains were associated with the cell membrane, whereas 90% were found in the cytoplasm. A similar degree of internalization occurred when the cells were incubated in the presence of NH₄Cl. After 2 h at 37°C, 3.5% of the silver grains were associated with the plasma membrane and 96.5% with the cytoplasm. In the presence of concanavalin A (conA), a plant lectin previously shown to block the cellular internalization of diphtheria toxin [14], the majority of the silver grains (61%) remained associated with the plasma membrane after incubation at 37°C.

Effect of antibody addition on NH₄Cl-mediated protection

Recent work has suggested that ammonia and amines prevent the clustering and subsequent internalization of receptor-bound epidermal growth factor and α₂-macroglobulin [15, 16]; similarly there are indications that NH₄Cl-mediated protection from diphtheria toxin results from a block of internalization of biologically significant toxin molecules [9, 10, 17]. Ivins et al. [9] showed
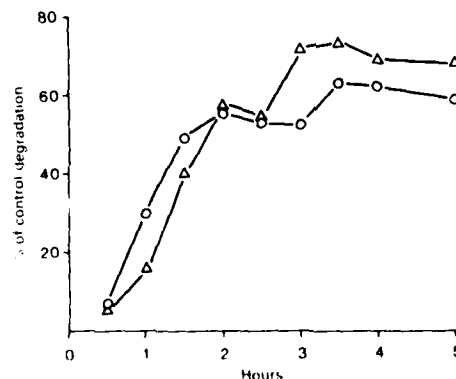
Fig. 6. Effect of antibody addition on NH_4Cl-mediated protection. ▲, Control; ☐, NH_4Cl plus antibody added at 0 time; ■, NH_4Cl only; ●, NH_4Cl plus antibody added at 60 min; ○, antibody only added at 60 min.

that NH_4Cl protects HEp-2 cells from diphtheria toxin only when NH_4Cl incubation is followed by neutralization with specific antitoxin. The implication is that NH_4Cl acts by maintaining the toxin in a position accessible to antibody, presumably on the cell surface. The data shown in fig. 6 indicate that a similar effect is exerted in the Vero system. In these experiments, cells were preincubated with diphtheria toxin for 18 h at 0°C, rinsed to remove unbound toxin, and warmed to 37°C in fresh medium with or without NH_4Cl. Specific antibody was added either simultaneously with the NH_4Cl (zero time) or 60 min after NH_4Cl addition. After 2 h at 37°C, the cells were rinsed, incubated a further 2 h, and assayed for protein synthesis. The results show that the cells were completely protected from diphtheria toxin (up to 100 ng/ml) when NH_4Cl and specific antibody were added simultaneously at zero time (line A). In the absence of specific antibody, NH_4Cl was only partially protective (40% inhibition of protein synthesis at 0.1 ng/ml of diphtheria toxin, as opposed to 90% in the control) (line B). When specific antibody was added 60 min after NH_4Cl, however, the protec-

tive effect was increased at least 100-fold (line C). This suggests that NH_4Cl does maintain a substantial fraction of the potentially lethal toxin in a position accessible to antibody neutralization. Antibody alone added at zero time completely protected the cells from toxin concentrations up to 10 ng/ml; a partial inhibition of protein synthesis was observed at 100 ng/ml. Antibody alone added at 60 min had no measurable protective effect (line D). Addition of NH_4Cl during the preincubation period (—18–0 h) had no effect on results; thus, dose-response curves identical with those in fig. 6 were obtained when NH_4Cl was present during the preincubation with toxin as well as during the period subsequent to the wash.

Time of addition of NH_4Cl

The data presented in fig. 7 show that NH_4Cl added to cells up to 60 min after toxin addition still exerts a protective effect. In these experiments, diphtheria toxin at the listed concentrations was added to
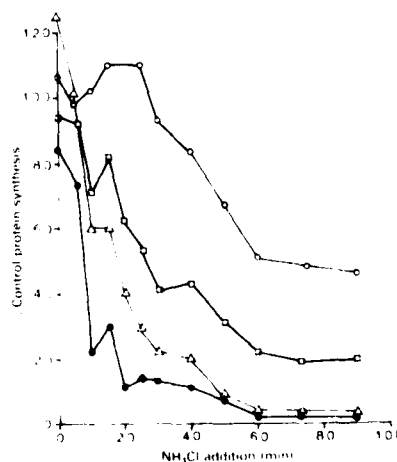

Fig. 7. Effect of time of NH_4Cl addition on diphtheria toxin-induced inhibition of protein synthesis. Toxin conc.: ○, 0.001; ☐, 0.01; △, 0.1; ●, 0.5 μg/ml.

cells at 37°C. NH₄Cl (10 mM) was added either simultaneously with toxin or at various times after toxin addition. The degree of protection was dependent on toxin concentration and the effect decreased with time over the period of the experiment. In all cases, the cells were completely protected by addition of NH₄Cl at zero time. At saturating concentrations of toxin, NH₄Cl-mediated protection could be detected up to 30 min after toxin addition; at lower concentrations of toxin, the effect was observable up to 50 min after toxin treatment.

DISCUSSION

The experimental evidence presented here demonstrates that NH₄Cl effectively protects Vero cells from the cytotoxic action of diphtheria toxin without blocking bulk uptake and degradation of labeled toxin molecules. This is in contrast to results obtained in other systems. Certain experiments with diphtheria toxin are consistent with the notion that NH₄Cl acts by preventing internalization at the level of the plasma membrane [9, 10, 17]. For example, when diphtheria toxin was incubated with cells in the presence of NH₄Cl, it was found that toxin was maintained in a position accessible to antitoxin neutralization, presumably at the cell surface [9]. Furthermore, recent studies have suggested that NH₄Cl and certain primary amines block the cellular internalization of α₂-macroglobulin and epidermal growth factor [15, 16], possibly by inhibiting a transglutaminase-like enzyme. It was hypothesized that a transglutaminase-like enzyme mediates the clustering of ligand–receptor complexes into 'coated pits' prior to endocytic internalization. An analogous explanation for the toxin-protective action of NH₄Cl does not

appear to obtain in our system, since we have shown that a number of acknowledged transglutaminase inhibitors, most notably dansylcadaverine and bacitracin, have no effect on either internalization or biological activity of diphtheria toxin (R. B. Dorland & S. H. Leppla, unpublished data).

Similarly our results can be interpreted to indicate the existence of dual internalization or dual intracellular processing mechanisms. We have shown that NH₄Cl has no detectable effect on either toxin internalization as determined by PIHP assay (fig. 4) or toxin degradation, as determined by release of TCA-soluble radioactivity into the culture medium (fig. 5). Both of these processes were shown by other methods to correspond to the biologically relevant uptake mechanism: the plant lectin, concanavalin A, was shown to prevent simultaneously toxin internalization and toxin-induced cell killing [14]; similarly, the lysosomotropic agent, chloroquine, was shown to block both toxin degradation and cytotoxicity [4]. However, the data in fig. 6 imply that the NH₄Cl-sensitive pathway is a minor subset of this major internalization and degradation process. Despite continuing active uptake of toxin by the cells, in the presence of NH₄Cl, a fraction of potentially lethal toxin molecules remains accessible to antibody neutralization for at least 60 min at 37°C. This suggests that NH₄Cl either affects the initial toxin internalization steps at the level of the plasma membrane or acts on some intracellular vesicular pool in rapid equilibrium with the plasma membrane.

Recent studies by Draper & Simon support the latter view: their experiments demonstrated that following incubation of cells with diphtheria toxin in the presence of NH₄Cl, antitoxin was protective if added at 37°C, but not if added at 4°C [18]. It

was suggested that a simple interpretation of this phenomenon indicates that NH_4Cl does not simply arrest the toxin at the cell surface, but directs toxin movement to a (vesicular?) compartment which either (*a*) is accessible to antitoxin at 37 but not at 4°C; or (*b*) recycles to the cell surface at physiological temperatures. Precedent for the putative recycling phenomenon is found in the recent studies using anti-plasma membrane immunoglobulins [19]. Alternatively, NH_4Cl may indeed trap toxin at the cell surface: the possibility that the shift to 4°C prevents the toxin–antibody interaction by some mechanism (e.g., eliciting a structural change in the toxin molecule, masking antigenic sites on the toxin molecule with membrane components) cannot be ruled out.

The data in fig. 7 demonstrate that at 37°C, NH_4Cl continues to exert a protective effect when added to cells after various periods of exposure to toxin. It appears that NH_4Cl is effective only during the period of active toxin uptake; i.e., toxin entering the cell in the presence of NH_4Cl does so nonproductively. At saturating concentrations of diphtheria toxin, NH_4Cl is effective when added up to 30 min after toxin addition. Previous work has shown that diphtheria toxin-treated cells cease active toxin uptake within 1–1½ h of toxin addition [1]; furthermore, it is known that the half-time of internalization of surface-bound toxin is approx. 25 min [2]. This suggests that once a certain proportion of potentially active toxin has entered the cell or reached a certain point in the processing pathway, NH_4Cl cannot prevent or reverse the expression of cytotoxicity.

Intracellularly, it has been shown that NH_4Cl inhibits the degradation of proteins in lysosomes, presumably by increasing the intralysosomal pH [20]. Previous results

from this laboratory demonstrated that diphtheria toxin appears to undergo a lysosomal degradation step, implying that some form of lysosomal activation may be essential for expression of biological activity [4]. Although NH_4Cl had no detectable effect on toxin degradation as measured by the appearance of TCA-soluble radioactivity in the culture medium, it is possible that the process is altered in some manner such as to prevent the generation or cytoplasmic release of enzymatically active fragments.

In conclusion, we have shown that NH_4Cl does not detectably affect binding, internalization, or degradation of ^{125}I-labeled diphtheria toxin by Vero cells. Results may indicate two pathways of cellular internalization: a non-productive bulk pathway and a productive minor pathway, inhibited by NH_4Cl. It seems more likely, however, that toxin is internalized by a single mechanism into an intracellular vesicular pool. The endocytic vesicles may then be targeted to the lysosomes, where degradation occurs, or may enter some other cytoplasmic traffic pattern, such that toxin becomes available for antibody neutralization. One possibility may be a 'recycling' of such vesicles to the cell surface. It appears that in the presence of NH_4Cl both processes occur, but without productive entry of fragment A into the cytoplasm.

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